BLADE-ON-PETIOLE1 Encodes a BTB/POZ Domain Protein Required for Leaf Morphogenesis in Arabidopsis thaliana

Chan Man Ha 1,2,3,4, Ji Hyung Jun 2,3,4, Hong Gil Nam 2,5 and Jennifer C. Fletcher 1,3,5

1 Department of Plant and Microbial Biology, University of California, Berkeley, Berkeley, CA 94720, U.S.A.
2 Division of Molecular Life Science, Pohang University of Science and Technology, San 31, Hyoja-dong, Pohang, Kyungbuk 790-784, Korea
3 Plant Gene Expression Center, USDA/UC Berkeley, 800 Buchanan Street, Albany, CA 94710, U.S.A.

The BLADE-ON-PETIOLE1 (BOP1) gene of Arabidopsis thaliana is required for proper leaf morphogenesis. BOP1 regulates leaf differentiation in a proximal-distal manner, and represses the expression of three class I knotted-like homeobox (knox) genes during leaf formation. Utilizing a map-based approach, we identified the molecular nature of the BOP1 gene, which encodes a BTB/POZ domain protein with ankyrin repeats. BOP1 is a member of a small gene family in Arabidopsis that includes the disease resistance regulatory protein NPR1. Insertions in and around BOP1 cause distinct lesions in leaf morphogenesis, revealing complex regulation of the locus. BOP1 transcripts are initially detectable in embryos, where they specifically localize to the base of the developing cotyledons near the SAM. During vegetative development, BOP1 is expressed in young leaf primordia and at the base of the rosette leaves on the adaxial side. During reproductive development, BOP1 transcripts are detected in young floral buds, and at the base of the sepals and petals. Our results indicate that BOP1 encodes a putative regulatory protein that modulates meristematic activity at discrete locations in developing lateral organs. This is the first report on a plant protein that plays a key role in morphogenesis with the distinctive combinatorial architecture of the BTB/POZ and ankyrin repeat domains.

Keywords: Ankyrin repeat — Arabidopsis thaliana — BOP1 — BTB/POZ — Differentiation — Meristem.

Introduction

Leaves are the sites of photosynthesis and are the main carbon source for higher plants. Leaves develop as lateral organs from the growing shoot tip, the shoot apical meristem (SAM). In Arabidopsis thaliana, leaf initiation occurs through coordinated changes in the rates and planes of cell division in a small group of founder cells on the SAM periphery (Steeves and Sussex 1989). Following initiation, leaf morphogenesis proceeds with regional patterning of the primordia along the proximal-distal, adaxial-abaxial and central-lateral axes (Engstrom et al. 2004). Establishment of leaf polarity along these three axes begins shortly after organ initiation, and involves developmental gradients that partition the leaf into distinct morphological and anatomical domains. During the final stages of leaf development, cell and tissue specification occurs through coordinated processes of cell division, expansion and differentiation. The genetic regulation of these different stages of leaf formation is under intense investigation, and a number of loci involved in leaf morphogenetic events have been identified.

The earliest molecular marker for Arabidopsis leaf initiation is the down-regulation of transcripts from the class I knotted-like homeobox (knox) gene SHOOTMERISTEMLESS (STM) from the incipient lateral primordia. STM is expressed throughout the SAM, and is required for the establishment and maintenance of the meristem cell population (Jackson et al. 1994, Long et al. 1996). STM fulfills this function in part through the negative regulation of ASYMMETRIC LEAVES1 (AS1) in the meristem apex, which restricts AS1 transcription to developing leaf primordia (Byrne et al. 2000). AS1 encodes a Myb domain transcription factor that confers leaf founder cell identity (Byrne et al. 2000), and has been shown to interact in yeast with a LOB domain leguminous protein encoded by the AS2 locus (Iwakawa et al. 2002, Lin et al. 2003, Xu et al. 2003b). Loss-of-function as1 and as2 mutants form lobed leaves, occasionally bearing ectopic shoots, in which the STM-related, meristematic class I knox genes BREVIPEDICELLUS (BP) and KNAT2 are ectopically expressed (Byrne et al. 2000, 2003a).
Ori et al. 2000, Semiarti et al. 2001). Exclusion of knox gene activity from organ founder cells by AS1 and AS2 is therefore important in the acquisition of differentiated leaf cell fates. The mutual antagonism between AS1-AS2 and the knox genes therefore establishes a pathway that distinguishes leaf founder cells from SAM cells, which enables the spatial separation and tightly controlled regulation of leaf cell fate.

In addition to AS1 and AS2, the BOP1 locus plays a key role in regulating meristematic activity in Arabidopsis leaves through the control of class I knox gene expression (Ha et al. 2003). bop1-1 plants are characterized by the formation of ectopic blade outgrowths along the adaxial side of cotyledon and rosette leaf petioles. These structures are generated through prolonged and clustered cell division in the mutant petioles, and have been interpreted as the result of disrupted cellular differentiation along the proximal-distal leaf axis. Three class I knox genes, BP, KNAT2 and KNAT6, are ectopically expressed in bop1-1 leaves, indicating a role for BOP1 in establishing and/or maintaining the repression of genes that normally promote the meristematic state. Interestingly, the requirement for BOP1 to control meristematic activity is not restricted to leaves, since bop1-1 plants also display ectopic outgrowths on the stem and at the base of floral organs. Here, we report the cloning of the BOP1 gene and the nature of the BOP1 gene product, the characterization of bop1 alleles and the pattern of BOP1 mRNA expression in wild-type Arabidopsis tissues.

Results

Map-based cloning of the BOP1 gene

The BOP1 locus was previously mapped to the lower arm of chromosome 3, approximately 7.28 cM below the TT5 locus (Ha et al. 2003). To refine the BOP1 region, we analyzed 1,148 F2 progeny (2,296 chromosomes) that were homozygous for the bop1-1 allele, and detected tight linkage between the BOP1 and BGL1 loci. Cleaved amplified polymorphic sequence (CAPS) markers were then developed across the BACs F24I3 and F28O9 that cover the BGL1 locus. These markers were used to fine-map the BOP1 locus to an approximately 40 kb region between the CAPS markers F24-21 and F28-58 (Fig. 1A).

A complementation testing strategy was used to delimit the position of BOP1 within this region. Overlapping cosmids clones were transformed into bop1-1 mutant plants that had been four-times backcrossed from Ler into the Ws-2 background. bop1-1 plants transformed with cosmids 145A10 showed complemented phenotypes in the T1 and T2 generations, with 94% of T1 plants and 100% of T2 plants displaying a revertant phenotype (Fig. 1A, B). Cosmid 84G23, which overlapped with the 5’ end of 145A10, failed to complement bop1-1, indicating that the 3’ portion of the 145A10 cosmid contained the BOP1 locus. Four annotated genes are located within this region of the 145A10 cosmid (Fig. 1A). Transformation of additional cosmids DNA overlapping 145A10 revealed that cosmids SG22 and 66J5 failed to complement the bop1-1 mutation, while cosmid 100C24 was sufficient to confer a revertant phenotype. These data narrowed the candidates for BOP1 to a single annotated gene, At3g57130. Comparison of genomic DNA sequences revealed that At3g57130 contains a single nucleotide substitution in bop1-1 relative to wild-type Ler. This mutation alters the final nucleotide of the coding sequence, converting the stop codon to a tyrosine residue and leading to the insertion of four additional amino acids before a new stop codon is encountered (Fig. 2A). Taken together, these data demonstrate that At3g57130 corresponds to the BOP1 gene.

Characterization of bop1 alleles

The BOP1 gene consists of two exons and a single intron (Fig. 2A). Genomic sequence analysis revealed the presence of a TATA box and an in-frame stop codon upstream of the transcription start site. Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) delineated 215 bp of 5’ untranslated sequence and 211 bp of 3’ untranslated sequence, producing a 2.4 kb mature BOP1 transcript. Database searches revealed the presence of a locus on Arabidopsis chromosome 2 that is highly similar to BOP1 at the nucleotide and amino acid levels. This BOP1-like gene, At2g41370, also consists of two exons and a single intron, and shares approximately 82% nucleotide identity with BOP1.
Three additional *bop1* alleles have been identified that were generated by insertional mutagenesis. The *bop1*-2 allele is caused by a T-DNA insertion several kilobases upstream of the *BOP1* locus (K. Krolikowski and S. Hake, personal communication). The *bop1*-3 allele is caused by a T-DNA insertion 446 bp upstream of the translation start site, while the *bop1*-4 allele is caused by a T-DNA insertion into the second exon of *BOP1* (Fig. 2A).

The transcription levels of *BOP1* were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) to detect differences in expression between wild-type and *bop1* mutant plants. *BOP1* transcripts are undetectable in *bop1*-4 mutant plants using primers spanning the *BOP1* coding region, demonstrating that *bop1*-4 is a null allele for the locus. *BOP1* transcript levels are slightly reduced in *bop1*-3 mutant plants compared with the wild type. In this analysis we observed that the *BOP1* expression level is increased in *bop1*-1 plants compared with wild-type plants (Fig. 2B). We interpret this result as an indirect effect of an increase in *BOP1*-expressing leaf tissue (see below) in *bop1*-1 mutants.

We next examined the *bop1*-3 and *bop1*-4 leaf phenotypes and compared them with those of *bop1*-1 plants. Relative to wild-type Ler leaves (Fig. 3A), *bop1*-1 leaves are misshapen and display ectopic outgrowths along the petiole and the base

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**Fig. 2** *BOP1* gene structure and alleles. (A) Schematic representation of the *BOP1* locus, showing the *bop1*-1 mutation and the location of the T-DNA insertions in the *bop1*-3 and *bop1*-4 alleles. The exon/intron organization of At3g57130 and the deduced protein structure are shown. The translation start codon (ATG) is designated nucleotide +1, and the stop codon (TAG) is at nucleotide position +2192. At3g57130 contains two exons (box) and one intron (bar). White boxes indicate the untranslated region in the exons, and gray boxes indicate the translated regions. The transcription start sites (~215) were determined by RACE-PCR. The *bop1*-1 mutant has a single G to C nucleotide substitution in the stop codon, which results in the addition of four amino acids to the predicted protein. Arrows indicate the locations of primers used for RT-PCR analysis. The BTB/POZ and ankyrin repeat domains of the *BOP1* protein are indicated. (B) *BOP1* transcription in *bop1* mutant plants. Total RNA isolated from the indicated 2-week-old plants was analyzed using RT-PCR to monitor the transcript accumulation of *BOP1*. Each PCR amplification of cDNA was conducted in parallel with specific primer sets for the *BOP1* coding region (F and R) and for TUBULIN4 as a control.

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**Fig. 3** Comparison of the *bop1* leaf phenotypes. (A) Wild-type Ler rosette leaf. (B) *bop1*-1 rosette leaf. (C) *bop1*-3 rosette leaf. (D) *bop1*-4 rosette leaf. (E) *bop1*-1/+ rosette leaf. (F) Higher magnification of the ectopic outgrowth from the *bop1*-1/+ leaf shown in (E). (G) *bop1*-1/*bop1*-3 rosette leaf. (H) *bop1*-1/*bop1*-4 rosette leaf. The shape of the rosette leaf reflects the severity of the *Bop1* phenotype. Arrows indicate the presence of ectopic outgrowths.
of the blade (Fig. 3B). *bop1-*3 plants, which have slightly reduced *BOP1* transcript levels, have a weak phenotype. This phenotype consists of a single ectopic outgrowth from the leaf petiole region in approximately 2–3% of the homozygous plants (Fig. 3C). Plants carrying the *bop1-*4 RNA null allele also have a weak phenotype, displaying a single ectopic leaf outgrowth and mild leaf lobing in a proportion similar to that in *bop1-*3 plants (Fig. 3D). Examining *bop1-*1 in the heterozygous state, we determined that approximately three out of four *bop1-*/+ plants formed a small ectopic outgrowth on one or two of their rosette leaves (Fig. 3E, F). This suggests that the *bop1-*1 allele is slightly semi-dominant with respect to leaf morphogenesis. Finally, we examined the *bop1-*1/*bop1-*3 and *bop1-*1/*bop1-*4 allele combinations and found that both combinations produced leaf phenotypes that were in the range of those observed in *bop1-*1 homozygotes (Fig. 3G, H). Thus, the severity of the *bop1* leaf phenotypes does not simply correlate with the level of *BOP1* transcription in the various *bop1* alleles.

**Fig. 4** Sequences similar to *BOP1*. (A) Amino acid sequence of the *BOP1* gene product. The BTB/POZ domain is shaded, the ankyrin repeat region is underlined and the two stretches of histidines are boxed. (B) Amino acid alignment of the BTB/POZ domain in *BOP1* and related plant proteins. (C) Amino acid alignment of the ankyrin repeat region. The alignment was performed using the CLUSTALW program. Asterisks indicate identical amino acids, colons indicate the conservative amino acid substitutions and dots indicate semi-conservative amino acid substitutions.

Structure of *BOP1* and related proteins

The *BOP1* gene encodes a predicted protein of 467 amino acids with a mass of 51.8 kDa. The predicted *BOP1* protein contains several distinct motifs that have been characterized in other plant and animal proteins. A BTB/POZ motif is located in the N-terminal region from residues 17 to 160, and an ankyrin repeat domain is located in the C-terminal region from residues 242 to 368 (Fig. 4). The four consecutive ankyrin repeats are indicated by long arrows above the sequence. Protein accession numbers: *BOP1*, NP_191272; *At2g41370*, NP_181668; Os*BOP1*, P0466H10; Le*NML1*, AAT57638; *NnNML1*, AAT57641; Le*NML2*, AAT57639; NPR1, AAM65726; RTP2, NP_568989.
length of the proteins, but contains 29 additional amino acids. OsBOP1 is 74% similar and 67% identical to Arabidopsis BOP1, although OsBOP1 lacks the two stretches of histidine residues that are present at the carboxyl terminus of Arabidopsis BOP1. OsBOP1 also contains a 24 amino acid insertion in the BTB/POZ domain (Fig. 4B) and a nine amino acid insertion in the first ankyrin repeat (Fig. 4C) that are absent from BOP1 and the other BOP1-like proteins in our analysis.

The combinatorial BTB/POZ and ankyrin repeat domain structure is conserved between BOP1 and the Arabidopsis regulatory protein NPR1 (NONEXPRESSOR OF PR GENES1) (Cao et al. 1997, Ryals et al. 1997), also known as NIM1 (Fig. 4B, C). NPR1 controls the onset of systemic acquired resistance (SAR) to a broad spectrum of pathogens, which is normally established after a primary exposure to avirulent pathogens (reviewed in Pieterse and Van Loon 2004). BOP1 shares 47% amino acid similarity and 30% identity with NPR1 across the full length of the proteins. BOP1 also aligns with NML (NIM-like protein) sequences that have been identified in several plant species, for example sharing 33% amino acid identity and 49–52% similarity with the NML1 and NML2 proteins from Lycopersicon esculentum. Three other uncharacterized Arabidopsis genes also encode proteins that are predicted to contain both a BTB/POZ domain and ankyrin repeats. The predicted gene products of At4g19660 and At5g45110 share 31% amino acid identity with BOP1, and At4g26120 is 30% identical across the length of the proteins. The RPT2 protein, which is involved in phototropnic signal transduction (Sakai et al. 2000), contains a BTB/POZ domain that shares similarity with BOP1, but lacks an ankyrin repeat domain.

Expression pattern of BOP1

To determine the sites of BOP1 activity, we examined the distribution of BOP1 transcript accumulation in wild-type and bop1-1 mutant plants. We isolated total RNA from 14 different tissue types and stages of development, and analyzed the spatial and temporal expression of BOP1 by semi-quantitative RT-PCR. BOP1 transcripts are detected at high levels in vegetative shoot apices, rosette leaves and floral buds, and at lower levels in stem nodes and mature flowers (Fig. 5A). Because the phenotypes of bop1-1 plants indicated an important role for BOP1 in rosette leaf morphogenesis (Ha et al. 2003), we analyzed BOP1 expression temporally in both young and mature rosette leaves, and spatially in the petiole and blade domains. BOP1 appears to be transcribed at the same relative level in whole rosette leaves regardless of age, but is expressed at very high levels in the petiole relative to the blade (Fig. 5A). This is consistent with the bop1-1 phenotype in which ectopic outgrowths develop more readily from the petioles of the rosette leaves. BOP1 is expressed at barely detectable levels in cotyledons and below the level of detection in cauline leaves, despite the observation that bop1-1 plants can form ectopic outgrowths on these organs (see below, Ha et al. 2003). BOP1 transcripts are also barely detectable in roots, and are not detected in stem internode tissue, flower pedicels and siliques (Fig. 5A). In bop1-1 mutant plants, BOP1 transcription is elevated in all tissues compared with the wild type and becomes readily detectable in cotyledons and cauline leaves (Fig. 5B), consistent with the manifestation of phenotypes in these tissues.

Next, we analyzed the specific pattern of BOP1 gene expression in wild-type tissues throughout Arabidopsis development. The first stage at which BOP1 transcripts can be reliably detected is the torpedo stage of embryogenesis, when BOP1 is found in two discrete foci at the base of the developing cotyledons, specifically on the adaxial side near the boundary with the shoot apical meristem (Fig. 6A). This expression pattern remains unchanged as the embryos mature (Fig. 6B). After germination, BOP1 transcripts are not observed in the SAM, but are present in the rosette leaf primordia initiating from its flanks (Fig. 6D). As the leaves grow out, BOP1 is expressed strongly yet in a highly restricted pattern at the base of the leaf primordia on the adaxial side adjacent to the SAM (Fig. 6E). This BOP1 leaf expression pattern mimics the cotyledon expression pattern. BOP1 transcripts are also present along the margin of young leaves, where the adaxial and abaxial domains juxtapose (Fig. 6F). In maturing leaves, a low level
expression becomes detectable across the adaxial portion of the leaf (Fig. 6D, F).

*bop1-1* mutant plants form ectopic outgrowths at the base of sepals and petals, indicating that the gene plays a role in flower development (Ha et al. 2003), and indeed *BOP1* shows a highly restricted expression pattern during the reproductive phase. *BOP1* is absent from the inflorescence meristem but is expressed in early floral buds (Fig. 6H). In floral meristems and maturing flowers, *BOP1* mRNA is specifically localized at the base of the developing sepals and petals, beginning as early as stage 3 (Fig. 6H, J). In mature flowers, *BOP1* continues to be transcribed at the base of the sepals and petals (Fig. 6K). No expression is detected at any stage using a *BOP1* sense probe, except in the leaf stipules (Fig. 6C, G, I). In sum, the vegetative and reproductive expression pattern of *BOP1* correlates very precisely with the tissues in which phenotypes are detected in *bop1-1* mutant plants (Ha et al. 2003).

**Discussion**

We have cloned the *BOP1* gene using a map-based strategy, and shown that it encodes a protein with a BTB/POZ domain and ankyrin repeats that is expressed in a highly restricted pattern during *Arabidopsis* development. Although there are 93 BTB/POZ-containing proteins and more than 240 ankyrin-repeat-containing proteins encoded in the *A. thaliana* genome alone, only a handful of proteins from *Arabidopsis* and other higher plants contain both domains. *BOP1* represents the second plant protein with this type of combinatorial protein architecture to be functionally characterized, and the first that plays a key role in morphogenesis.

**Functional domains of the BOP1 protein**

The amino terminus of *BOP1* shows strong similarity to proteins containing a BTB/POZ domain. The BTB/POZ domain is an evolutionarily conserved ~120 amino acid region that was originally named for the *Drosophila* Broad-Complex, Tramtrack and Bric-a-brac proteins (BTB) (Zollman et al. 1994), and for two of the main classes of proteins in which it can be found, Pox virus and zinc finger proteins (POZ) (Bardwell and Treisman 1994). The BTB/POZ domain is conserved throughout eukaryotes, but has undergone independent expansion in plants and different animal lineages (Aravind and Koonin 1999). Three-dimensional structural analysis of the BTB/POZ domain shows that it has two distinct parts. The amino-terminal half forms a four-stranded β-sheet with two associated short α-helices, while the carboxyl-terminal portion forms two α-helices separated by an extended region (Aravind and Koonin 1999). The BTB/POZ domain has been shown to mediate protein homodimerization or multimerization.
null mutant plants, which have a mild phenotype, make no detectable full-length BOP1 mRNA. The deficiency of BOP1 protein in the null mutant may be largely complemented by the activity of the BOP1-like protein encoded by At2g41370 (Fig. 4). In contrast to bop1-4 null mutants, bop1-1 plants have a severe leaf phenotype and increased levels of BOP1 expression. These contradictory observations can be explained by the specific expression pattern of BOP1 at the base and the margin of wild-type leaves. The primary leaves of bop1-1 mutants form ectopic leaf outgrowths, each of which would be expected to express BOP1 at the margin and base. Thus many more cells express BOP1 in bop1-1 plants than in wild-type plants, accounting for the increased expression at the level of the whole plant.

Our results indicate that the originally characterized bop1-1 allele confers a weakly semi-dominant phenotype in Arabidopsis rosette leaves, as bop1-1 heterozygous plants have a very weak phenotype (Fig. 3E). The semi-dominant phenotype of bop1-1 could be derived from a haploid deficiency in the heterozygous plants or from a dominant-negative interaction between the mutant allele and the wild-type allele. Since the mutant phenotype of homozygous bop1-1 plants is more severe than that of bop1-4 null mutant plants, haplo-insufficiency is unlikely to be the reason for the semi-dominance of the bop1-1 allele. Thus, we suggest that the mutant BOP1-1 protein may interfere with the normal function of other proteins in the leaf morphogenesis pathway. Mutant BOP1-1 protein, possibly in an altered conformational state due to addition of the C-terminal amino acids, could, for instance, bind to one or more of its regular interaction partners and titrate away its normal function in the cell. A precedent for this hypothesis is the demonstration that chromosomal translocations of the BTB/POZ proteins PLZF and BCL6 result in novel fusion proteins that act in a dominant-negative fashion by sequestering their partner proteins into inactive multimeric complexes (Bardwell and Treisman 1994).

The formation of simple leaves by Arabidopsis plants requires the down-regulation of class I knox genes such as STM, BP and KNAT2, since ectopic expression of one or more of these genes in developing leaves causes leaf lobing and compound leaves. Our results indicate that BOP1 is necessary to prevent the formation of ectopic, lobed blades along the adaxial side of cotyledons and rosette leaves, and to negatively affect the expression of BP, KNAT2 and KNAT6 in Arabidopsis leaves (Ha et al. 2003). Given that the BOP1 protein contains several motifs that mediate protein-protein interactions, we hypothesize that BOP1 may exert its regulatory effects in leaf primordia by interacting with other leaf-expressed gene products through its BTB/POZ and/or its ankyrin repeat domains.

Possible mechanisms of BOP1 activity

In analyzing the available bop1 mutants, we observed that the level of BOP1 transcription in the various bop1 alleles does not appear to correlate with phenotype severity. bop1-4 RNA null mutants, which have a mild phenotype, make no
of the bop1-4 null mutant. Other candidates for leaf-specific co-acting factors include both AS1 and AS2, since BOP1 appears to function synergically with these two proteins to control leaf morphogenesis. AS1 and AS2, like BOP1, prevent the ectopic expression of class I knox genes in lateral organ primordia (Byrne et al. 2000, Ori et al. 2000, Semiarti et al. 2001). Thus, these three proteins might act together in a complex that represses knox gene transcription in leaves. However, bop1-1 plants have some cotyledon and leaf phenotypes that are not observed in as1 or as2 plants, suggesting that at least a subset of BOP1 functions is independent of these two genes. Another potential BOP1-interacting protein is the product of the LATERAL ORGAN BOUNDARIES (LOB) gene, which is expressed in a pattern similar to BOP1 in developing leaves (Shuai et al. 2002). Thus BOP1 might interact with LOB or some of the related LBD proteins (Shuai et al. 2002).

The combinatorial architecture of the BOP1 protein suggests several possible mechanisms through which BOP1 might regulate leaf morphogenesis. The BTB/POZ domain has been proposed to act as a scaffold for the organization of higher-order cellular structures, such as the cytoskeleton, ubiquitin ligase substrate complexes and chromatin. Some animal BTB/POZ proteins, such as BCL6 and PLZF, interact with transcriptional co-repressors that can recruit histone deacetylases for chromatin remodeling at the target loci (Hyuhn and Bardwell 1998, Lin et al. 1998). More recently, other BTB/POZ proteins have been shown to act as substrate-specific adaptor proteins for Cullin3 E3 ubiquitin ligases in yeast and Caenorhabditis elegans (Geyer et al. 2003, Pintard et al. 2003, Xu et al. 2003a).

Based on the BCL6 and PLZF protein paradigm, one hypothesis is that BOP1 might act as a transcriptional repressor of class I knox gene expression in leaf primordia. Through its BTB/POZ domain, BOP1 could potentially recruit histone deacetylases or other chromatin remodeling proteins to the BP, KNAT2 and KNAT6 promoters to lock them in a transcriptionally inactive state as leaf maturation progresses. Alternatively, BOP1 may act in a proteasome-mediated degradation pathway that targets one or more meristem-promoting factors, or meristem gene-activating factors, for turnover in developing leaves. The ankyrin repeats could mediate either the recruitment of the interaction partners, or the stability of the resulting protein complexes. Future biochemical experiments to determine the interaction partners of BOP1 will help to distinguish between these possibilities.

Materials and Methods

Plant material and growth conditions

The bop1-3 allele (SALK_012994) was obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (Columbus, OH, U.S.A.). The bop1-4 allele (386G09) was obtained from the Genome Analysis of the Plant Biological System project (GABI-Kat, Cologne, Germany). Seeds were imbibed at 4°C for 3 d before sowing and were grown in a greenhouse under long days (16 h light and 8 h dark) with a day/night temperature cycle of 22°C/18°C.

For the genetic analysis of F1 plants, we reciprocally crossed bop1-1 mutant plants with Ler, and with bop1-3 and bop1-4 mutants. A total of 76 F1 plants between bop1-1 and Ler, 13 F1 plants between bop1-1 and bop1-3, and 12 F1 plants between bop1-1 and bop1-4 were observed for the ectopic outgrowths phenotype.

Map-based cloning

The bop1-1 mutation was mapped using cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel 1993). bop1-1 mutant plants in the Ler ecotype were crossed with the Columbia (Col) ecotype. DNA for mapping studies was prepared from 1,148 individual F2 progeny plants with mutant phenotypes. Additional CAPS markers for fine mapping were developed using Arabidopsis genome sequence data (TAIR, http://www.Arabidopsis.org). The BOP1 nucleotide and deduced protein sequences were used for database searches using the BLAST network service (http://www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequences of BOP1 and homologous proteins were aligned using CLUSTAL W, version 1.8 (Thompson et al. 1994). Alignment of the ankyrin repeats was performed using the consensus sequence described in Sedgwick and Smardon (1999). CAPS marker sequences developed for this study are available upon request.

Complementation of bop1-1

The cosmids clones that contain the BOP1 genomic regions were obtained from the Genomic Arabidopsis Resource Network (GARNet; John Innes Centre, Norwich, UK). Overlapping cosmids clones of GARNet transformed into Agrobacterium (GV3101 strain), and in turn used to transform bop1-1 mutants using the floral dip method (Clough and Bent 1998). T1 seeds were plated on media supplemented with 40 µg ml⁻¹ kanamycin (Sigma, St. Louis, MO, U.S.A.). Kanamycin resistance and rescued phenotypes were observed after 2 weeks.

RACE-PCR

Total RNA from 12-day-old Ler seedlings was prepared using RNeasy kit (Qiagen, Valencia, CA, USA). RACE-PCR cDNA was synthesized using GeneRacer™ Kit (Invitrogen, Carlsbad, CA, U.S.A.). The 5′ end of the cDNA was determined by 5′-RACE with primer RBI-5(1) (5′-ATCTAAAGAGATCGACGGCT-GCA-3′) and a GeneRacer™ 5′ primer. The 3′ end sequence of the cDNA was determined by 3′-RACE with primer RBI-3(1) (5′-GTGAT-GGGAGAAGGACTCAATCTAGA-3′) and a GeneRacer™ 3′ primer. PCR product was cloned into the pcR®-2.1-TOPO® vector (Invitrogen) and sequenced.

RT-PCR

For the analysis of Ler wild-type plants, shoot apices, cotyledons, rosette leaf petioles, rosette leaf blades, young rosette leaves and mature rosette leaves were collected 17 d after planting; roots were collected 13 d after planting; cauleine leaves, inflorescence stem nodes, open flowers, pedicels and green siliques were collected 35 d after planting. For the genetic analysis of F1 plants, we reciprocally crossed bop1-1 mutant plants with Ler, and with bop1-3 and bop1-4 mutants. A total of 76 F1 plants between bop1-1 and Ler, 13 F1 plants between bop1-1 and bop1-3, and 12 F1 plants between bop1-1 and bop1-4 were observed for the ectopic outgrowths phenotype.
Blc: 5′-AGAGGGCATGAAAGATTTGAGA-3′). Primers to amplify the Arabidopsis TUBULIN4 gene were used as an internal control (Ha et al. 2003).

In situ hybridization

For probe generation, the full-length BOP1 cDNA sequence was amplified using BOP1-specific primers (BI-IS1: 5′-GTCGCAGCTCTCTCTTCTCTACCT-3′; BI-IS2: 5′-ACAGTTAAATGATATGTTAGATG-3′) and cloned into the SacI and SphI site of the pBluescript KS+ plasmid (Stratagene, La Jolla, CA, U.S.A.). T3 and T7 polymerase (Promega, Madison, WI, U.S.A.) were used to synthesize sense and antisense UTP-digoxigenin-labeled RNA as described (Jackson 1992). Plant fixation and in situ hybridization were performed as described (Jackson 1992).

Acknowledgments

We acknowledge the Arabidopsis Biological Resource Center, the Genome Analysis of the Plant Biological System project and the Salk Institute for Biological Studies for providing insertion lines, and Genomic Arabidopsis Resource Network for cosmid clones. This work was supported by a fellowship from the Postdoctoral Fellowship Program of the Korea Science and Engineering Foundation (KOSEF) to C.M.H., a grant from the Crop Functional Genomics Frontier Research Program of the Ministry of Science and Technology of Korea to H.G.N. and a United States Department of Agriculture CRIS grant to J.C.F.

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(Received September 3, 2004; Accepted September 15, 2004)